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AN NADPH-INDUCED CHANGE IN LIPID BILAYER OF RAT LIVER MICROSOMES AS OBSERVED BY SPIN-LABELED PHOSPHATIDYLCHOLINE

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SUMMARY: Lipid bilayer of rat liver microsomes was spin-labeled by incubating with liposomes of 1-acyl 2-(12-doxylstearoyl) glycero-3-phosphorylcholine. When NADPH was added to the labeled microsomes, there appeared a rapidly tumbling component of spin label in the EPR spectrum. NADH was less effective than NADPH. The appearance of the sharp signal was prevented under anaerobic conditions or in the presence of either carbon monoxide, phenyl isocyanide or cytochrome c. The appearance of the rapidly tumbling component in the EPR spectrum was found to be due to the release of spin moiety from the membrane into the aqueous phase. That the release was associated with superoxide anion formation or with lipid peroxidation is unlikely, since 1) superoxide dismutase had little effect, 2) addition of either α-tocopherol or EDTA did not inhibit the release. These observations suggest that electron transfer from NADPH to oxygen via cytochrome P-450 system induces a physical perturbation in the lipid bilayer resulting in the release of its component into the aqueous phase.

INTRODUCTION

A number of studies have been performed concerning the relationship between microsomal membrane proteins and phospholipids composing lipid bilayer of the membrane (1, 2). For instance, it has long been known that various reactions catalyzed by microsomal cytochrome P-450 system are dependent on the presence of phospholipids (3, 4). More recently, Stier and co-workers (5, 6) suggested the possibility that phospholipid molecules in the immediate vicinity of cytochrome P-450 were more highly organized than the bulk of membrane phospholipids. In an effort to observe changes provoked on phospholipids during cytochrome P-450 reaction, we have prepared microsomes labeled with spin-labeled phosphatidylcholine. In this paper, it is reported that the spin moiety of the label embedded in rat liver microsomes is released

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into the aqueous phase during electron transfer from NADPH to oxygen via the cytochrome P-450 system.

MATERIALS AND METHODS

1-Acyl 2-(12-doxylstearoyl) glycero-3-phosphorylcholine was synthesized by acylation of egg lysophosphatidylcholine with anhydride of 12-doxylstearic acid according to the method of Hubbel and McConnell (7). The spin label (15 mg) was suspended in 5 ml of 0.1 M KCl-0.05 M potassium phosphate buffer (pH 7.4) and sonicated. Microsomes were prepared from livers of male rats (6 to 8 weeks old), and suspended in 0.1 M potassium phosphate buffer. The spin-labeled phosphatidylcholine liposomes (3 mg) were incubated with rat liver microsomes (30 mg of protein) in 0.1 M potassium phosphate buffer for 60 min at 37 °C and centrifuged at 105,000 x g for 80 min. The spin-labeled microsomal pellets were washed once, and suspended in 0.1 M potassium phosphate buffer or 0.1 M Tris-HCl buffer (pH 7.4) to give a final protein concentration of 16 mg/ml.

A spin-labeled microsomal suspension (8 mg protein per ml, about 0.05 ml) was sucked up into a quartz tube (internal diameter, 0.7 mm), the end of which was sealed by parafilm, and the tube was set in a holder of a Varian E-12 EPR Spectrophotometer. All EPR spectra were obtained at room temperature (22 °C) with microwave power 20 mW, microwave frequency 9.1 GHz, modulation frequency 100 kHz and modulation amplitude 2 G.

Protein concentrations were determined by the method of Lowry et al. (8), with bovine serum albumin as the standard. NADPH, NADH and NADP$^+$ were products of Oriental Yeast Co. Phenyl isocyanide was synthesized by the conventional method. Superoxide dismutase purified from bovine erythrocytes was a gift from Drs. Hiwatashi and Sugiyama. All other chemicals were of the highest purity available from commercial sources.

RESULTS

The resonance spectrum of the labeled microsomes was that of a typical membrane embedded spin-labeled phosphatidylcholine (Fig. 1, curve 1). A small amount of rapidly tumbling component was always present, although its ratio to the major component varied with preparations. The addition of NADPH to the spin-labeled microsomes induced a remarkable change in the resonance spectrum (Fig. 1). As the figure shows, the rapidly tumbling component became clearer and increased in size. Under these conditions, no significant loss of the signal due to nonspecific reduction of the nitroxide radical was observed. NADH induced a much slower spectral change. The addition of NADPH to the unlabeled microsomes gave no EPR spectrum, and no change in the spectrum was observed when the compound was added to the spin-labeled phospholipid liposomes. The addition of NADP$^+$ was also without effect on the spectrum of spin-labeled microsomes.

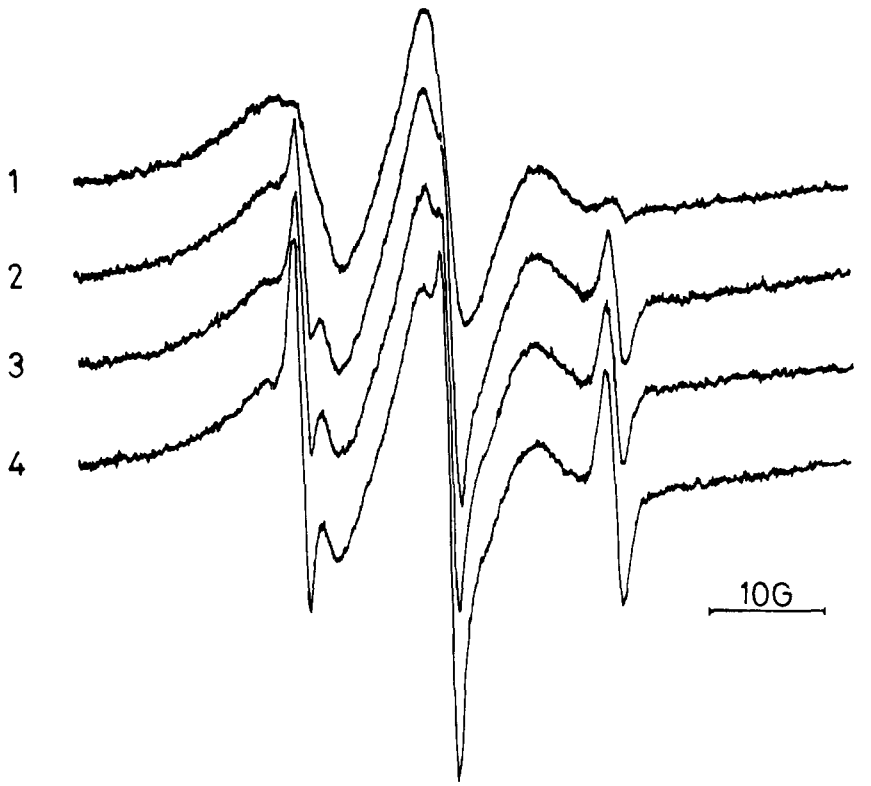

Figure 1. Effect of NADPH on the spin-labeled microsomes. To the spin-labeled microsomes (8 mg/ml in 0.1 M potassium phosphate buffer, pH 7.4) in a test tube, NADPH (final concentration, 5 mM) was added and the mixture was incubated at 22 °C under aerobic conditions. At various time intervals, aliquots were taken for EPR measurements. EPR spectra of (1) 0 min, (2) 5 min, (3) 10 min, and (4) 25 min after addition. Scanning was from left to right at a rate of 100 G/4 min.

To test the possibility that the microsomal electron transfer system is involved in causing the EPR spectral change, the effect of various inhibitors of microsomal electron transport on the appearance of sharp signal was investigated. As shown in Fig. 2, the addition of cytochrome c prevented the appearance. It was also inhibited in the presence of either carbon monoxide or phenyl isocyanide. The signal change was not observed under anaerobic conditions. These findings strongly suggest that the EPR spectral change is closely associated with the electron transfer from NADPH to oxygen via cytochrome P-450.

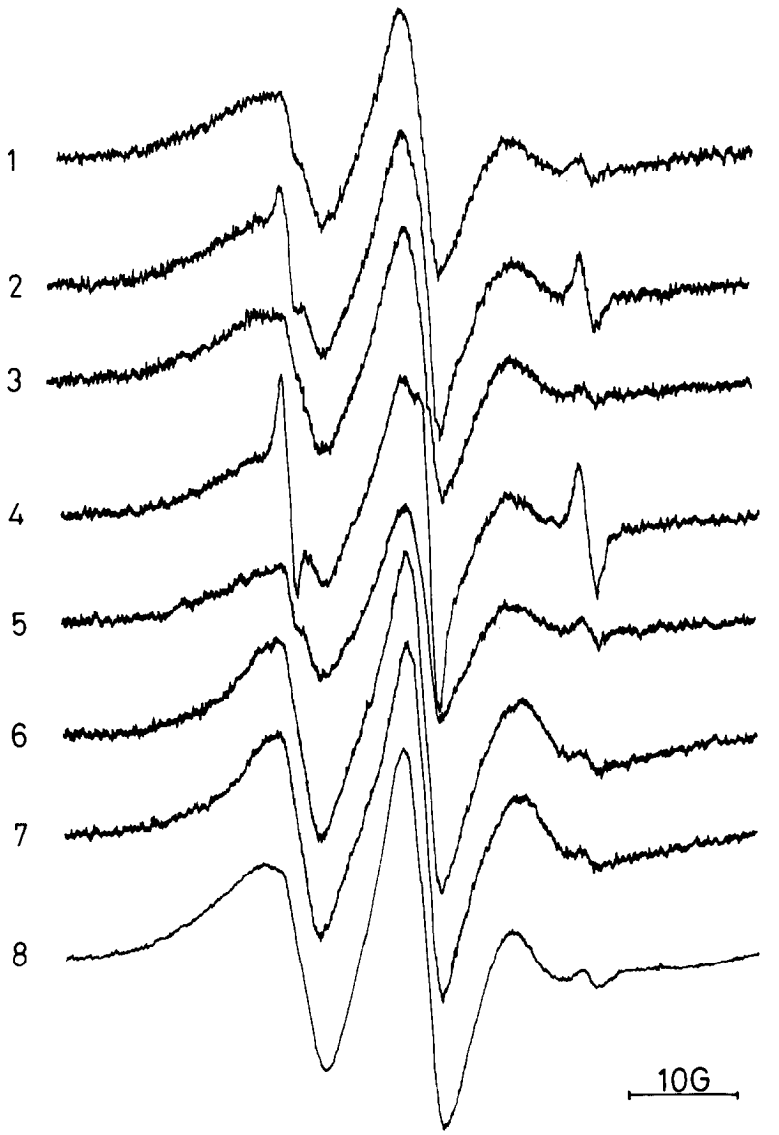

Figure 2. Effect of inhibitors of microsomal electron transfer system on the spin-labeled microsomes. The EPR spectra were recorded 14 min after the additions of following compounds. (1) 0 time control, (2) NADPH (0.25 mM), (3) cytochrome c (0.5 mM) followed by NADPH (0.25 mM), (4) NADPH (5 mM), (5) carbon monoxide bubbling followed by NADPH (5 mM), (6) phenyl isocyanide (1 μg), (7) phenyl isocyanide (1 μg) followed by NADPH (5 mM), (8) the spin-labeled microsomes were degassed and NADPH (5 mM) was added.

Experiments were carried out to examine whether superoxide anion or lipid peroxidation was involved in the appearance of the rapidly tumbling component in the EPR spectrum. The addition of an excess amount of super-

oxide dismutase (30 mg/ml) or EDTA (10 mM) had little effect on the appearance of sharp signal. Further, the same spectral change was observed with α-tocopherol-treated microsomes (α-tocopherol, 25 mg/g liver wet weight, was added during homogenization). These results seem to dismiss the involvement of superoxide anion or lipid peroxidation as a possible explanation for the phenomenon.

After aerobic incubation of spin-labeled microsomes with NADPH, the membrane fraction was recovered by centrifugation. The membrane fraction showed an EPR spectrum similar to that of the original microsomes before NADPH addition except that the peak heights were decreased, while the supernatant showed only the sharp signal (Fig. 3). These results indicate that spin label is released into aqueous phase during the reaction with NADPH. Little degeneration of electron transfer system appears to occur during this treatment, since the membrane fraction responded again to NADPH with the appearance of the rapidly tumbling component in EPR spectrum.

DISCUSSION

The functional properties of microsomal cytochrome P-450 system are known to be intimately related to the microenvironment provided by membrane phospholipids (3-6). Therefore, it will naturally be expected that the phospholipids composing the microenvironment of this enzyme system would in turn, be physically perturbed during the enzyme reactions. The experiments presented in this paper were attempted to explore the latter point. The results clearly suggest that the microsomal electron transfer from NADPH to oxygen induces a physical perturbation of the lipid bilayer resulting in the release of its component into the aqueous phase.

Superoxide anion formation and lipid peroxidation, both of which are known to take place during the oxidation of NADPH by microsomes (9, 10), do not seem to be involved in this phenomenon.

A quantitative estimate of the released spin moiety was made from the peak height of high field signal using spin-labeled stearic acid in aqueous

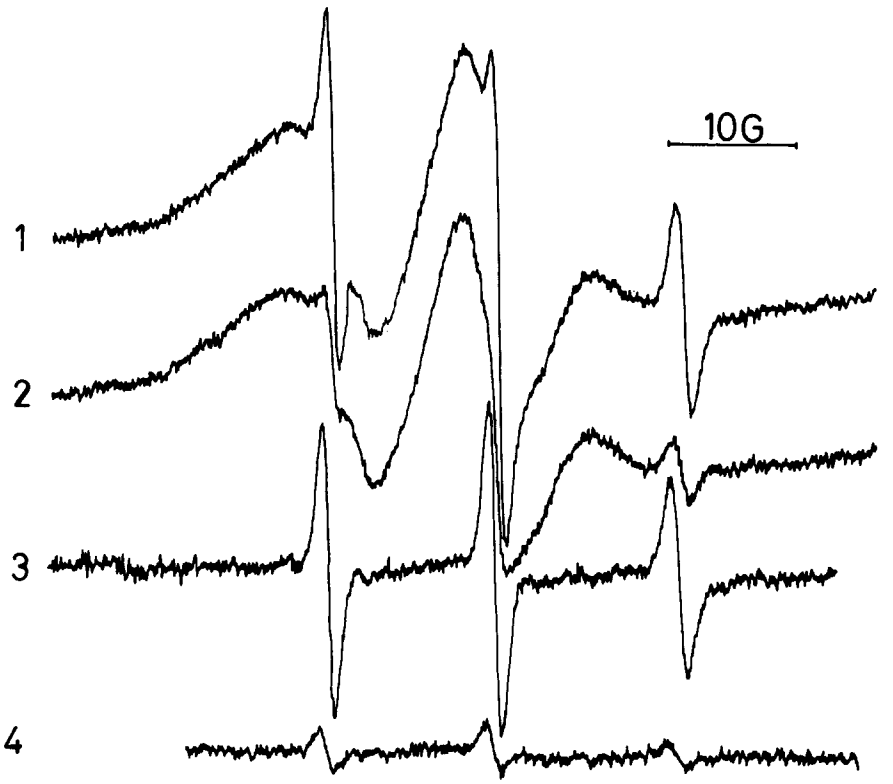

Figure 3. The rapidly tumbling component of EPR spectrum was due to the spin moiety released into the aqueous phase. To the spin-labeled microsomes (16 mg in a final volume of 2 ml of 0.1 M Tris-HCl buffer) in a test tube, 10 μmol NADPH were added and the mixture was incubated at 30 °C for 16 min under aerobic conditions. After incubation, the volume of the reaction mixture was adjusted to 7 ml and centrifuged at 105,000 x g for 80 min. The pellets were suspended in 2 ml of the same buffer. As a control experiment, the spin-labeled microsomes were similarly treated except that no NADPH was added. EPR spectra of:(1) incubation mixture with NADPH (total volume, 2 ml; recorder gain, 3,200); (2) pellets (total volume, 2 ml; recorder gain, 3,200); (3) supernatant (total volume, 7 ml; recorder gain, 6,300); and (4) control supernatant (total volume, 7 ml; recorder gain, 6,300).

solution as the standard. According to a rough estimation, about 2.4 % of total spin quantity appeared as the sharp signal during 60-min incubation period of microsomes with NADPH.

The spin-labeled rat liver microsomes showed a resonance spectrum consisting of two components. The major component was of a typical spectrum of lipid bilayer, and the minor was the spectrum of freely tumbling species. It is uncertain whether the minor component was due to the spin moiety released from the membrane spontaneously during the preparation. In contrast

to the rat liver microsomes, the spin-labeled membrane of rabbit liver microsomes or bovine adrenal mitochondria did not show this minor rapidly tumbling component (Okamoto, M. and Miura, R., unpublished observation). Furthermore, these spin-labeled membranes did not respond to NADPH or NADH with the appearance of sharp signal as in the rat liver microsomes. These observations might suggest a peculiar nature of rat liver microsomes with regard to the susceptibility of membrane phospholipids to the physical perturbation.

As to the chemical identity of the released substance(s), evidence was obtained that it is not the intact phosphatidylcholine, but the decomposition product(s) thereof. The microsomes were labeled with 1-acyl 2-([1-^{14}C]stearoyl) glycero-3-phosphorylcholine, and incubated with or without NADPH. The supernatant, obtained by centrifugation, from the incubation mixture containing NADPH had a radioactivity significantly higher than that from the control incubation mixture without NADPH. A TLC analysis on the organic extracts of these supernatants showed that radioactive substance(s) with a Rf value similar to that of hydroxystearic acid appeared specifically in the supernatant derived from incubation with NADPH. The chemical identification of the substance(s) is in progress.

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